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**HYDROGEN PEROXIDE AND B-NICOTINAMIDE ADENINE DINUCLEOTIDE  
SENSING AMPEROMETRIC ELECTRODES BASED ON ELECTRICAL CONNECTION  
OF HORSERADISH PEROXIDASE REDOX CENTERS TO ELECTRODES THROUGH A  
THREE-DIMENSIONAL ELECTRON RELAYING POLYMER NETWORK**

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involves two known steps. In the first step, NAD(P)H transfers two electrons and a proton to a dissolved quinoid. The quinoids are typically derived of phenazines, however phenothiazine and phenoxazine derivatives are also useful. In the second step, two electrons and a proton are transferred from the reduced quinoid to  $O_2$ . This reaction produces  $H_2O_2$  and the original quinoid. Because the two reactions are quantitative, the sensitivity and the linear range of the resulting NADH and NADPH electrodes are identical with those of the  $H_2O_2$  electrode,  $1Acm^{-2}M^{-1}$  and  $1 \times 10^{-7}M - 2 \times 10^{-4}M$  respectively.

**Abstract:**

Hydrogen peroxide is efficiently electroreduced at an electrode modified with a hydrophilic, permeable film of horseradish peroxidase (HRP) covalently bound to a 3-dimensional epoxy network having polyvinyl pyridine (PVP)-complexed  $[\text{Os}(\text{bpy})_2\text{Cl}]^{+3/+2}$  redox centers. The sensitivity of the resulting  $\text{H}_2\text{O}_2$  cathode at 0.0V(SCE) is  $1\text{Acm}^{-2}\text{M}^{-1}$ . Its current increases linearly with  $\text{H}_2\text{O}_2$  concentration in the  $1 \times 10^{-7}\text{M} - 2 \times 10^{-4}\text{M}$  range. Related NAD(P)H cathodes are based on stoichiometric homogeneous reduction of  $\text{O}_2$  to  $\text{H}_2\text{O}_2$  by NADH or NAD(P)H. The reduction involves two known steps. In the first step, NAD(P)H transfers two electrons and a proton to a dissolved quinoid. The quinoids are typically derived of phenazines, however phenothiazine and phenoxazine derivatives are also useful. In the second step, two electrons and a proton are transferred from the reduced quinoid to  $\text{O}_2$ . This reaction produces  $\text{H}_2\text{O}_2$  and the original quinoid. Because the two reactions are quantitative, the sensitivity and the linear range of the resulting NADH and NADPH electrodes are identical with those of the  $\text{H}_2\text{O}_2$  electrode,  $1\text{Acm}^{-2}\text{M}^{-1}$  and  $1 \times 10^{-7}\text{M} - 2 \times 10^{-4}\text{M}$  respectively.

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### *Introduction:*

Selective electrooxidation of NADH and NADPH co-factors (reaction 1) of



enzymes allows, in principle, amperometric assay of a substantial number of biochemicals. When the electrooxidation products are the cofactors  $\text{NAD}^+$  or  $\text{NADP}^+$ , these can be enzymatically re-reduced and electrocatalytic enzyme electrodes can be made. The reversible potential of the  $\text{NADH}/\text{NAD}^+$  couple is  $-0.56\text{V}$  (SCE) at  $\text{pH}7$ .<sup>1</sup> Because, the reaction involves the concerted transfer of two electrons and a proton, it is usually slow, proceeding at practical rates on most electrodes only at high overpotentials. At these high overpotentials reaction products of NADH and other constituents of biological fluids that are also electrooxidized interfere with the amperometric assays of  $\text{NAD(P)H}$ .<sup>2,3</sup> Following Elving's definition of this problem,<sup>4-6</sup> several groups, particularly those of Miller,<sup>7-10</sup> Gorton<sup>11</sup> and Kulys,<sup>12,13</sup> developed electrodes on which reaction 1 proceeds rapidly at low overpotentials. The most successful electrodes, of which Gorton's phenoxazine-derivative and Kulys' phenazine based electrodes are examples,<sup>11-16</sup> utilize electrode-bound, electrode-adsorbed or freely diffusing mediators having quinoid structures in their oxidized state. The quinoids effectively catalyze reaction 1 at potentials near  $0.0\text{V}$ (SCE).

Here we consider a more complex but nevertheless very fast and efficient set of coupled reactions for the amperometric assay of NAD(P)H. The first is a homogeneous solution reaction, exemplified by 2, where, as in the



electrode reactions of Miller, Kulys, and Gorton, two electrons and a proton are transferred from NAD(P)H to a quinoid mediator. A particularly effective mediator is water-soluble 5-methyl-phenazonium cation ( $\text{PMS}^+$ ) which is quantitatively reduced by NAD(P)H to 5-methylphenazine (PMSH). PMSH is next reoxidized to  $\text{PMS}^+$  by dissolved molecular oxygen which is, in turn, reduced to  $\text{H}_2\text{O}_2$  (reaction 3).



With reactions 2 and 3 being quantitative, each mole of NAD(P)H produces one mole of  $\text{H}_2\text{O}_2$ .  $\text{H}_2\text{O}_2$  is then assayed through electroreduction on the "wired" peroxidase electrode (reaction 4).



Several previously reported detection schemes for NADH and NADPH have utilized reactions 2 and 3, amperometrically sensing the depletion of oxygen<sup>17,18</sup> or spectrophotometrically measuring the  $\text{H}_2\text{O}_2$  generated.<sup>19-21</sup> We now add to these amperometric reduction of  $\text{H}_2\text{O}_2$  on peroxidase, electrically connected ("wired") through a permeable 3-dimensional redox polymer network to an electrode. Several horseradish peroxidase modified

diffusionally mediated and mediatorless type electrodes have been earlier described. Their characteristics are compared with the "wired" HRP electrode in Table 1.

In the "wired" HRP electrodes electrons from the electrode are relayed to the enzyme through a redox epoxy network to which the enzyme (HRP) is covalently bound. The centers consist of  $[\text{Os}(\text{bpy})_2\text{Cl}]^{3+/2+}$ , complexed to polyvinyl pyridine. The HRP and the redox polymer are crosslinked into a 3-dimensional epoxy network with a water-soluble diepoxide. In earlier papers we showed that the resulting redox epoxy accepts electrons from substrate-reduced enzymes relaying these to electrodes.<sup>22,23</sup> Here we show that the network also relays electrons in the reverse direction from the electrode to a bound enzyme. Network-bound HRP is efficiently electroreduced at 0.0V (SCE), and  $\text{H}_2\text{O}_2$  is detected with  $1 \text{ A cm}^{-2} \text{ M}^{-1}$  sensitivity. Because NAD(P)H concentrations are stoichiometrically translated to  $\text{H}_2\text{O}_2$  through reactions 2 and 3, these cofactors are also detected at the same potential with the same sensitivity.

#### *Experimental:*

**Reagents:** Horseradish peroxidase (HRP) E.C. 1.11.1.7 (Sigma P-8375 type VI, 260 Units/mg) was used. Poly (ethylene glycol 600 diglycidyl ether), tech. grade (PEGDGE) was purchased from Polysciences (cat. # 8211). The osmium redox polyamine (Polymer I, fig. 1) was synthesized as described previously.<sup>24</sup> NADH and NADPH were purchased from Sigma (340-110 and N-1630 respectively). 5-methylphenazonium

methylsulfate was from Aldrich. Other mediators were from Aldrich or Sigma. All chemicals were used as received.

**Electrode Construction and Preparation:** Rotating disk electrodes were made of 1 cm length of 3mm diameter vitreous carbon rods from Atomergic Chemicals Corp. These were press-fitted into one end of a Teflon sleeve. The opposite end of the sleeve had a press-fitted stainless steel rod threaded to match a Pine rotator. Electrical contact between the vitreous carbon and stainless steel rods was made with a silver epoxy Epo-tek H20E from Epoxy Technology Inc.. The electrodes were polished first with 6 $\mu$ m then with 1 $\mu$ m diamond suspension, followed by 0.3 $\mu$ m alumina. The polishing compounds were from Buehler. After each polishing step, the electrodes were sonicated 3 to 6 min. in deionized water.

**Enzyme Immobilization:** HRP (2mg) was dissolved in 100 $\mu$ L 0.1M sodium bicarbonate solution. After the addition of 50  $\mu$ L of 12 mg/mL sodium periodate, the enzyme solution was incubated in the dark for 2.3 hours. A 10mg/mL solution of Polymer I was used to dilute aliquots of the enzyme solution to make enzyme:Polymer I solutions of various ratios (1:5, 1:10, 1:50, 1:100). A 1 $\mu$ L loading of enzyme:Polymer I solution was applied to the polished vitreous carbon surface. The electrodes were allowed to partially dry for 5 to 15 min., after which, 1 $\mu$ L of a 1 mg/mL solution of PEGDGE was applied. The electrodes were then cured in water-saturated air at room temperature for > 4 h.

Electrodes were also made by coimmobilizing the  $\text{NaIO}_4$  oxidized HRP with a polyamine that had no redox centers. This polyamine was obtained by reacting polyvinyl pyridine (PVP) (MW 60,000) with 2-bromoethylamine to form the pyridinium-N-ethylamine derivative. It is thus similar to Polymer I, but has no  $[\text{Os}(\text{bpy})_2\text{Cl}]^{3+/2+}$  redox centers. The HRP was crosslinked to the polyamine using PEGDGE through the above described process.

**Buffers, Electrodes and Electrochemical Equipment:** The electrodes were operated at room temperature in modified Dulbecco's buffer (PBS) pH 7.4. Unless otherwise indicated, the solutions were well aerated. All mediator solutions were made daily and protected from light until used. Potentials were referenced to a saturated calomel electrode from EG & G cat.# K0077. A platinum wire was used as the counter electrode. The chronoamperometric experiments were performed on an EG & G potentiostat/galvanostat model 173 and recorded on a Kipp & Zonen XY recorder model BD91. The cyclic voltammograms were run on an EG & G potentiostat/galvanostat model 273A and recorded on a P.C. with software developed in this lab. The rotator used was a Pine Instruments AFMSRX with the ACMDI 1906C shaft.

### *Results:*

**$\text{H}_2\text{O}_2$  Sensing Electrodes:** Electroreduction of  $\text{H}_2\text{O}_2$  is observed on electrodes modified with HRP immobilized in the epoxy network formed of either the polyamine without  $[\text{Os}(\text{bpy})_2\text{Cl}]^{3+/2+}$  redox centers (Figure 2) or with  $[\text{Os}(\text{bpy})_2\text{Cl}]^{3+/2+}$  redox centers (Figure 3). When there are no

$[\text{Os}(\text{bpy})_2\text{Cl}]^{3+/2+}$  centers in the polymer, reduction takes place at potentials negative of 0.2V (SCE). In  $1 \times 10^{-4}$  M  $\text{H}_2\text{O}_2$  a  $\sim 1 \mu\text{Acm}^{-2}$  plateau is reached near 0.1V (SCE). In the redox epoxy network formed by PEGDGE crosslinking of polymer I containing  $[\text{Os}(\text{bpy})_2\text{Cl}]^{3+/2+}$  centers, the current density at 0.0V (SCE) increases by two orders of magnitude to about  $100 \mu\text{Acm}^{-2}$ . Furthermore,  $\text{H}_2\text{O}_2$  electroreduction is observed already at +0.45V(SCE) and the steady-state current plateaus at +0.3V(SCE). (Figure 4)

The dependence of the catalytic  $\text{H}_2\text{O}_2$  electroreduction current density on the HRP:Polymer I ratio in PEGDGE crosslinked films is seen in Figure 5. The current density is nearly independent of the HRP:Polymer ratio at low ( $< 1 \times 10^{-4}$  M)  $\text{H}_2\text{O}_2$  concentration. At higher ( $> 1 \times 10^{-4}$  M)  $\text{H}_2\text{O}_2$  concentration the current density increases as the film becomes richer in HRP up to a ratio of 1:5; (Figure 5) the current density then decreases upon further increasing the enzyme content (not shown). The current densities of electrodes with 1:10 and the 1:5 (HRP:Polymer I) film ratios do not differ greatly. For electrodes with the 1:5 (HRP:Polymer I) films the sensitivity in the  $0-1 \times 10^{-4}$  M  $\text{H}_2\text{O}_2$  concentration range is  $1 \text{Acm}^{-2}\text{M}^{-1}$ , i.e. the current density at  $1 \times 10^{-4}$  M  $\text{H}_2\text{O}_2$  is  $100 \mu\text{Acm}^{-2}$ . When the  $\text{H}_2\text{O}_2$  concentration exceeds 0.25mM, the current is time dependent and decays because of (slow) substrate-inhibition of HRP. Control electrodes, made with PEGDGE crosslinked films of Polymer I without HRP show no measurable  $\text{H}_2\text{O}_2$  response.

Figure 6 shows Levich plots for 1:100 and 1:5 (HRP:Polymer I) electrodes in  $1 \times 10^{-4}$  M  $\text{H}_2\text{O}_2$ . Linear dependence of the current density on the square-

root of the angular velocity is observed only up to about 400 RPM. At higher angular velocities the current densities increase with the HRP content of the films, but are not proportional to the HRP content. At 2500 RPM increasing the HRP concentration from 1:100 to 1:5 increases the current density by only 30%.

The insensitivity of the electrodes to the partial pressure of oxygen is seen in Figure 7. There is no measurable difference between the calibration curves of the 1:100 (HRP:Polymer I) electrode in nitrogen-purged or air-saturated solutions. For the 1:5 (HRP:Polymer I) electrode there appears to be a marginal difference, with the readings in air exceeding those in nitrogen by less than 2%.

The dynamic range of the 1:5 (HRP:Polymer I) electrode is seen in Figure 8. The current density increases linearly with  $\text{H}_2\text{O}_2$  concentration over a range of three orders of magnitude from about  $1 \times 10^{-7} \text{M}$  to  $1 \times 10^{-4} \text{M}$  (correlation coefficient 0.997; slope  $1 \text{Acm}^{-2} \text{M}^{-1}$ ). At  $10^{-5} \text{M}$   $\text{H}_2\text{O}_2$  the 0 - 95% risetime is 2 min. At lower concentrations the risetimes are longer. Following an  $\text{H}_2\text{O}_2$  injection raising the concentration from 0.0M to  $1 \times 10^{-7} \text{M}$ , the risetime is about 10 min. The noise equivalent  $\text{H}_2\text{O}_2$  concentration is 3nM, i.e. at  $1 \times 10^{-8} \text{M}$   $\text{H}_2\text{O}_2$  the signal to noise ratio is 3. The background current, measured after the electrode was allowed to stabilize for 30 min, is  $70 \text{nAcm}^{-2}$ .

**NAD(P)H Sensing Electrodes Derived of HRP "Wired" to a 3-Dimensional Redox Polymer Network:** The "wired" HRP electrodes are insensitive to NAD(P)H, i.e. the background current at 0.0V (SCE) does not

change when either cofactor is added. However, if 1-methoxy-5-methylphenazonium methylsulfate (I), 5-methylphenazonium methylsulfate (II), Meldola's blue (III), Nile blue (IV), toluidine blue O (V), methylene blue (VI), thionin (VII), flavin mononucleotide (VIII), 4,5-dihydro-4,5-dioxo-1H-pyrol[2,3-f]quinoline-2,3,9-tricarboxylic acid (PQQ) (IX) or methylene violet (Bernthsen) (X) is added, an NAD(P)H concentration dependent cathodic current is observed. The structures of these heterocyclic quinoids are shown in Figure 9. The relative effectiveness of these mediators in the  $H_2O_2$  forming reaction is in the order of their listing, the phenazonium derivatives and Meldola's blue being the most effective and flavin mononucleotide, PQQ and methylene violet the least. Addition of any of the mediators at  $<10\mu M$  concentration does not produce a current response.

The dependence of the steady state current density on the NADH concentration for aerated solutions containing  $1.6\mu M$  5-methylphenazonium methylsulfate is seen in Figure 10. The dependence is linear through the  $1-100\mu M$  NADH concentration range and the slope, i.e. sensitivity, is  $1 A cm^{-2} M^{-1}$ , similar to that for  $H_2O_2$ . Corresponding results for NADPH are seen for the 1:5 electrode in Figure 11. The linear range for NADPH is from 1 to  $200\mu M$ , and the sensitivity is again  $1 A cm^{-2} M^{-1}$ . The equilibration times for steady state measurements depend on the concentration of the mediator; a higher mediator concentration results in acceleration of the  $H_2O_2$  production. Typically, the 0 - 95% risetime of the current following an NADH injection was 5-7 min. at  $3.3\mu M$  5-methylphenazonium methylsulfate concentration.

As expected from reaction 3, electroreduction currents were observed only in aerated or oxygenated solutions. The current did not increase when  $O_2$  rather than air was bubbled through the solution. When the solutions were purged of oxygen by bubbling of  $N_2$ , the current reversed, i.e. an electrooxidation current was observed in the PMSH ( $PMS^+$  and NADH) containing solution. Electrooxidation of PMSH proceeded on glassy carbon electrodes whether or not these were modified with HRP containing films. Even minimal aeration of the PMSH solutions reversed the current, but only on HRP modified electrodes.

**Light Effects:**  $PMS^+$  solutions strongly absorb  $\lambda < 480$  nm light. It has been reported that the mechanism of reduction of heterocyclic quinoid dyes by NADH can involve their excited states.<sup>17,25</sup> Furthermore, the oxidant of the NADH-reduced quinoid dye may not be ground state (triplet) oxygen but excited (singlet) oxygen, formed through energy transfer from the excited dye in its triplet state. Thus, as a precaution, the effect of  $0.2 \text{ mW cm}^{-2}$  4100° K color temperature "cool-white" fluorescent on the risetime of the current was checked. Control experiments with  $PMS^+$  ( $1 \times 10^{-5} \text{ M}$ ) show that the current is not changed when the electrode is operated in the dark or with the above ambient irradiance. It was also noted, however, that  $PMS^+$  measurably photodecomposed even at the low irradiance by the ambient light.

## Discussion

**HRP Based Hydrogen Peroxide Sensing Electrodes:** Table 1 compares  $H_2O_2$  electrodes based on direct, diffusionally mediated, and redox polymer-relayed electroreduction of HRP. Comparison of the electrodes shows that the wiring of HRP to an electrode, i.e. its covalent binding to a hydrophilic 3-dimensional electron-relaying redox network increases sensitivity. In the absence of osmium-complex relays the observed sensitivity,  $1 \times 10^{-2} \text{Acm}^{-2}\text{M}^{-1}$ , is two orders of magnitude lower than that in their presence (Figures 2 and 3). In the first case only redox centers of HRP molecules actually contacting the electrode surface may be electroreduced. These redox centers produce the redox wave in figure 2. In contrast, most HRP molecules in the films, the thickness of which is  $\approx 10^{-4} \text{cm}$ , are electrically accessible when electrons are relayed through  $[\text{Os}(\text{bpy})_2\text{Cl}]^{3+/2+}$  centers complexed to the polyvinyl pyridine backbone in Polymer I. Electrooxidation of HRP in the electron-relaying epoxy network starts at +0.45V i.e. 0.18V positive of the +0.27V redox potential of the  $[\text{Os}(\text{bpy})_2\text{Cl}]^{3+/2+}$  centers. This implies that oxidized HRP accepts electrons from the network even when the ratio of the reduced to oxidized centers is only about 1 to 1000.

The optimal HRP:Polymer I ratio in the film (Figure 5) is near 1:5. At higher enzyme content, the electron-relaying capacity of the films is diminished by the non-relaying HRP in the network. The network, with an electron diffusion coefficient below  $10^{-8} \text{cm}^2 \text{s}^{-1}$ ,<sup>24</sup> does not transport or transfer electrons to the bound enzyme molecules fast enough to match

their turnover rate at optimal ( $10^{-4}\text{M}$ ) substrate concentration. Had the electron transport through the polymer been faster, still higher current densities might have been realized. That the electrodes are limited by the rate of electron transfer either through the network or from the network to the enzyme is seen in the Levich plots of Figure 6. These show normal solution mass transfer limited kinetics of the substrate, characterized by linear dependence of the current density on the square root of the angular velocity, only at low angular velocities. At high angular velocities where the kinetics does not depend linearly on substrate mass transport and depend only weakly on enzyme content, the characteristics are dominated by transport of either electrons or substrate through the film. Previous work with glucose oxidase containing redox epoxy films suggests electron transfer or transport limitation.<sup>24</sup>

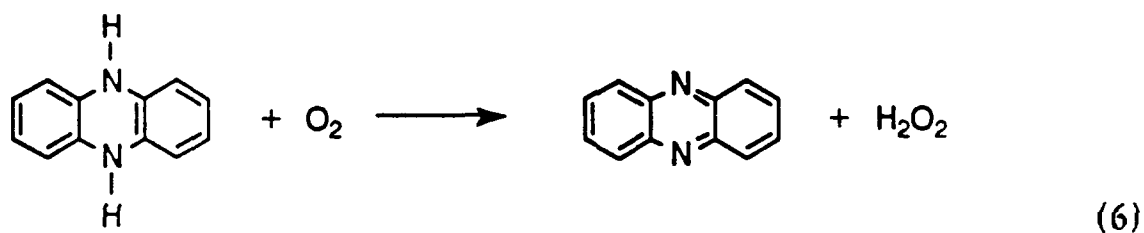
**Reduction of Heterocyclic Quinoids by Two Electron Plus Proton Transfer from NAD(P)H:** The earlier results of Miller, Gorton, Kulys and their colleagues<sup>26-31</sup> show that NAD(P)H is rapidly and cleanly oxidized to  $\text{NAD(P)}^+$  by transferring two-electrons plus a proton to any of a variety of dissolved or electrode-surface bound quinoids, including native quinoids on oxidized graphite (reaction 5).



5-methylphenazonium derivatives and Meldola's blue and its derivatives are particularly fast two-electron plus proton acceptors from NAD(P)H.

The homogeneous bimolecular two electron plus proton transfer rate from NADH to PMS<sup>+</sup> (reaction 2) is  $3.8 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$  at 25°C.<sup>32</sup>

Oxidation of PMSH by O<sub>2</sub> (reaction 3), whereby PMS<sup>+</sup> is recovered and H<sub>2</sub>O<sub>2</sub> is produced, has a bimolecular homogeneous rate constant of  $1.8 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$  in water at 25°C.<sup>32</sup> Thus in an aqueous solution in equilibrium with air ( $\approx 2.5 \times 10^{-4} \text{ M O}_2$ ), the oxidation of PMSH is rapid. Indeed the related reaction of dihydrophenazine and O<sub>2</sub> in an organic solvent (reaction 6) has been considered as an industrial process for the production of H<sub>2</sub>O<sub>2</sub>.<sup>33</sup>



The rates of reactions 2 and 3 and H<sub>2</sub>O<sub>2</sub> diffusion may all slow the response, i.e. risetime of the NAD(P)H sensor. At low NADH concentration (0.1  $\mu\text{M}$ ) the calculated rate from reaction 2 or inherent diffusion-controlled transport of H<sub>2</sub>O<sub>2</sub> are limiting factors of the electrodes kinetics. At higher NADH concentration (100  $\mu\text{M}$ ) and at low PMS<sup>+</sup> concentrations (1.6  $\mu\text{M}$ ) reaction 3 limits the electrode's response. The calculated H<sub>2</sub>O<sub>2</sub> formation rates through reaction 3 and experimental sensor risetimes are of the same order of magnitude.

The variation of current with concentration, and the  $1 \text{ A cm}^{-2} \text{ M}^{-1}$  sensitivity, for NADH (Figure 10) and NADPH (Figure 11) through their

$1 \times 10^{-7}$  to  $2 \times 10^{-4}$  concentration range is identical for that of  $\text{H}_2\text{O}_2$  (Figure 8). We infer from the identical sensitivities and dynamic ranges that the homogeneous two-electron and proton transfer reactions proceed either at or very close to unit current efficiency, i.e. that  $\text{NAD(P)H}$  produces a stoichiometric amount of  $\text{H}_2\text{O}_2$  through reactions 2 and 3. The actual mechanism of  $\text{H}_2\text{O}_2$  production involves more steps than represented by equations 2 and 3.

**Interferences:** Schmidt et al. suggested that, "Reduction of the oxygen proceeds by a complex sequence of reactions, producing among other intermediates the superoxide radical ion, which leads to hydrogen peroxide and this in turn is capable of oxidizing methylene blue and so a stoichiometric production of hydrogen peroxide is not observed."<sup>17</sup> The disparity probably results from the higher dye concentrations employed by Schmidt et al. At a high dye concentration the rates of side reactions, particularly between the reduced dye and  $\text{H}_2\text{O}_2$ , are increased. At the  $\text{NAD(P)H}$  and mediator concentrations employed here we observe only the stoichiometric reaction 7.



As is evident from Reaction 3, the assay requires that the solutions be aerated. A decrease in  $\text{O}_2$  partial pressure will slow reaction 3. Nevertheless, even in this case the ultimate steady state current will not change, because reaction 3 is irreversible. The high bimolecular rate constant ( $1.8 \times 10^2 \text{M}^{-1} \text{sec}^{-1}$ ) for PMSH oxidation by  $\text{O}_2$  usually ensures a rapid reaction in air-exposed solutions. When the oxygen concentration is

only 1/10th of that in a well aerated solution (a typical value at 25°C being 0.25mM), the half life of PMSH is 154 sec. assuming a pseudo first order reaction in PMSH.

HRP catalyzed reactions may cause severe interference by a number of interferants.  $\text{H}_2\text{O}_2$  oxidized HRP may be reduced by any of a number of hydrogen donors. Such reduction will cause loss of catalytic current. Addition of 0.1mM ascorbate, a common component of biological samples, will reduce the cathodic current by over 50%. Current will also be lost if NAD(P)H directly reduces  $\text{H}_2\text{O}_2$ -oxidized HRP. This reaction is actually observed in our experiments as a dip in the current from the electrodes when NAD(P)H is initially injected into a solution with a substantial  $\text{H}_2\text{O}_2$  concentration already present. Once the NAD(P)H reacts to form  $\text{H}_2\text{O}_2$  and NAD(P)<sup>+</sup>, the current recovers. The ultimate current is not lowered, because reactions occurring at the electrode surface do not change the bulk solution concentrations. The bulk  $\text{H}_2\text{O}_2$  concentration being reached through the homogeneous solution reactions 2 & 3. Beyond organic hydrogen donors,  $\text{H}_2\text{O}_2$  itself is oxidized by HRP to  $\text{O}_2$  and water.<sup>34</sup> Fortunately, the latter reaction is not fast.

### *Conclusions*

In contrast with redox centers of flavoprotein enzymes like glucose oxidase, that do not communicate directly with carbon electrodes on which the enzymes are adsorbed, redox centers of directly adsorbed horseradish peroxidase do communicate electrically with carbon electrodes.<sup>35,36</sup> The maximum current density does not exceed, however, in the absence of a

diffusional mediator, or of non-diffusing electron-relaying centers, the current density associated with the turnover of the enzyme layer directly contacting the electrode surface. Oxidized horseradish peroxidase molecules, that are remote from the electrode surface, do not accept electrons from electrodes unless the electrons are relayed through redox centers in the polymer. The current density is increased by two orders of magnitude when the HRP molecules bound throughout the thick film are connected to the electrode through its three-dimensional electron relaying network. The sensitivity of the resulting amperometric  $\text{H}_2\text{O}_2$  sensor is  $1\text{Acm}^{-2}\text{M}^{-1}$  at 0.0V(SCE) and its dynamic range is  $1\times 10^{-7}\text{M}$ - $2\times 10^{-4}\text{M}$   $\text{H}_2\text{O}_2$ .

Two-electron plus proton transfer from either NAD(P)H to quinoids produces stoichiometric concentrations of  $\text{H}_2\text{O}_2$ . With the NAD(P)H stoichiometrically translated to  $\text{H}_2\text{O}_2$ , their concentrations can be amperometrically assayed at "wired" horseradish peroxidase cathodes poised at 0.0V(SCE) (Figure 12). The sensitivities and dynamic ranges of these cathodes are identical with those of the  $\text{H}_2\text{O}_2$  cathode,  $1\text{Acm}^{-2}\text{M}^{-1}$  through the  $1\times 10^{-6}$ - $2\times 10^{-4}\text{M}$  concentration range. Although the assay of these co-factors requires molecular oxygen, the electrodes are not excessively sensitive to variations in  $\text{O}_2$  partial pressure because the quinoid catalyzed NAD(P)H reactions with  $\text{O}_2$  are irreversible.

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### **up coming research**

H<sub>2</sub>O<sub>2</sub> and NAD(P)H sensing electrodes having a sensitivity of 1 A cm<sup>-2</sup> M<sup>-1</sup> and a dynamic range of 3 orders of magnitude (10<sup>-7</sup> - 10<sup>-4</sup> M) are described. The sensing of NAD(P)H is based on its stoichiometric conversion to H<sub>2</sub>O<sub>2</sub>.

### Figure Captions

Figure 1: Composition of the electron relaying redox polymer ( $m = 1$ ;  $n = 3.35$ ;  $o = 0.6$ ). After crosslinking with PEGDGE, it forms a 3-dimensional network that is able to relay electrons to covalently bound HRP. The polymer is referred to as Polymer I.

Figure 2: Cyclic voltammogram of a vitreous carbon electrode modified with the Schiff base formed of polyaldehyde-HRP and Polymer I *without*  $[\text{Os}(\text{bpy})_2\text{Cl}]^{3+/2+}$  electron relaying centers, additionally crosslinked with PEGDGE. Aerated pH7 physiological phosphate buffer (PBS) solution; scan rate  $2.5\text{mVs}^{-1}$ ; 500 RPM. A: no  $\text{H}_2\text{O}_2$ ; B:  $0.1\text{mM H}_2\text{O}_2$ .

Figure 3: Electrode as in Figure 2, but *with*  $[\text{Os}(\text{bpy})_2\text{Cl}]^{3+/2+}$  electron relaying centers (Polymer I). 1:5 enzyme to polymer I ratio. Conditions for A and B as in Figure 2. A: no  $\text{H}_2\text{O}_2$ ; B:  $0.1\text{mM H}_2\text{O}_2$ . For C the scan rate is  $2.5\text{mVs}^{-1}$ ; 2000 RPM;  $0.5\text{mM H}_2\text{O}_2$ .

Figure 4: Potential dependence of the steady state current density for a vitreous carbon electrode modified with PEGDGE crosslinked HRP-Polymer I at 1:5 ratio. PBS, 1000 RPM,  $1 \times 10^{-5}\text{M H}_2\text{O}_2$ .

Figure 5: Dependence of the current density on the  $\text{H}_2\text{O}_2$  concentration for vitreous carbon electrodes modified with PEGDGE crosslinked HRP-Polymer I films. The HRP:Polymer I ratios are indicated. PBS;  $0.0\text{V}(\text{SCE})$ ; 1000 RPM.

Figure 6: Dependence of the current density on the square root of the angular velocity of PEGDGE crosslinked 1:5 and 1:100 HRP:Polymer I electrodes. PBS 0.0V(SCE); 0.1mM H<sub>2</sub>O<sub>2</sub>.

Figure 7: Steady-state calibration curves for PEGDGE crosslinked 1:5 and 1:100 HRP:Polymer I electrodes in air (solid circles) and nitrogen (open circles) 0.0V(SCE); PBS; 500 RPM.

Figure 8: Dynamic range of the 1:5 HRP:Polymer I electrode. Steady state measurements at 0.0V(SCE), 1000 RPM, PBS.

Figure 9: Structures of the mediators which are able to catalytically cycle through reactions 2 and 3. Note the central quinoidal structure stabilized by the adjacent aromatic rings. 1-methoxy-5-methyl-phenazonium methylsulfate (I), 5-methylphenazonium methylsulfate (II), Meldola's blue (III), Nile blue (IV), toluidine blue O (V), methylene blue (VI), thionin (VII), flavin mononucleotide (VIII), 4,5-dihydro-4,5-dioxo-1H-pyrol[2,3-f]quinoline-2,3,9-tricarboxylic acid (PQQ) (IX), methylene violet (Bernthsen) (X).

Figure 10: Dependence of the steady-state electrocatalytic reduction current density on the NADH concentration for PEGDGE cross-linked 1:5 and 1:100 HRP:Polymer I film-modified vitreous carbon electrodes. 0.0V(SCE), 1000 RPM, 1.6 $\mu$ M 5-methylphenazonium methylsulfate (PMS<sup>+</sup>) PBS.

Figure 11: Dependence of the steady-state electrocatalytic reduction current density on the NADPH concentration for the 1:5 electrode of Figure 10. Conditions as in Figure 10, except that the 5-methyl-phenazonium methylsulfate concentration was  $4.7\mu\text{M}$ .

Figure 12: Cycles of the proposed NADH (and NADPH) cathodes.

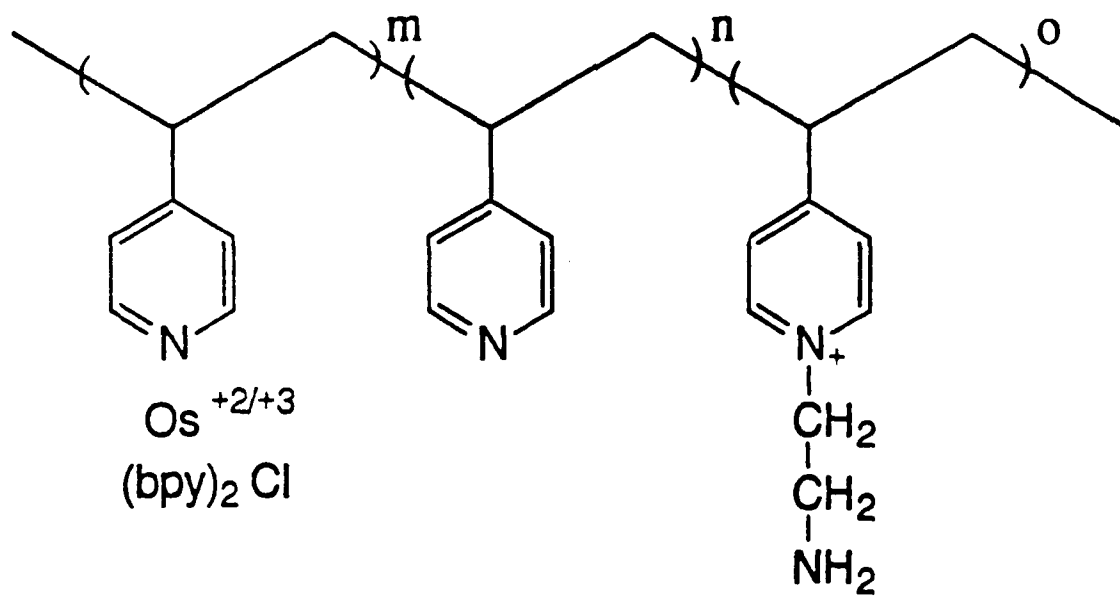
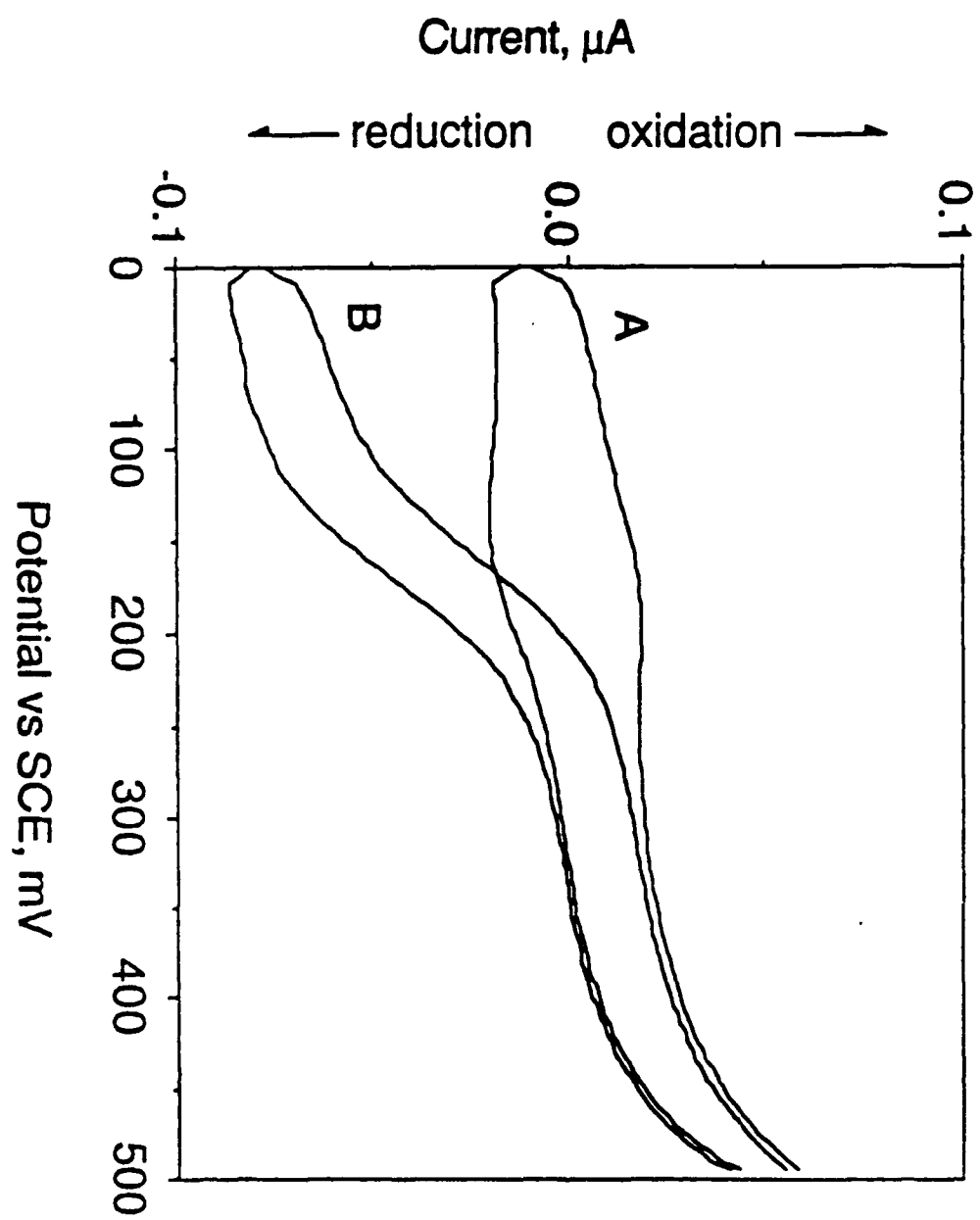
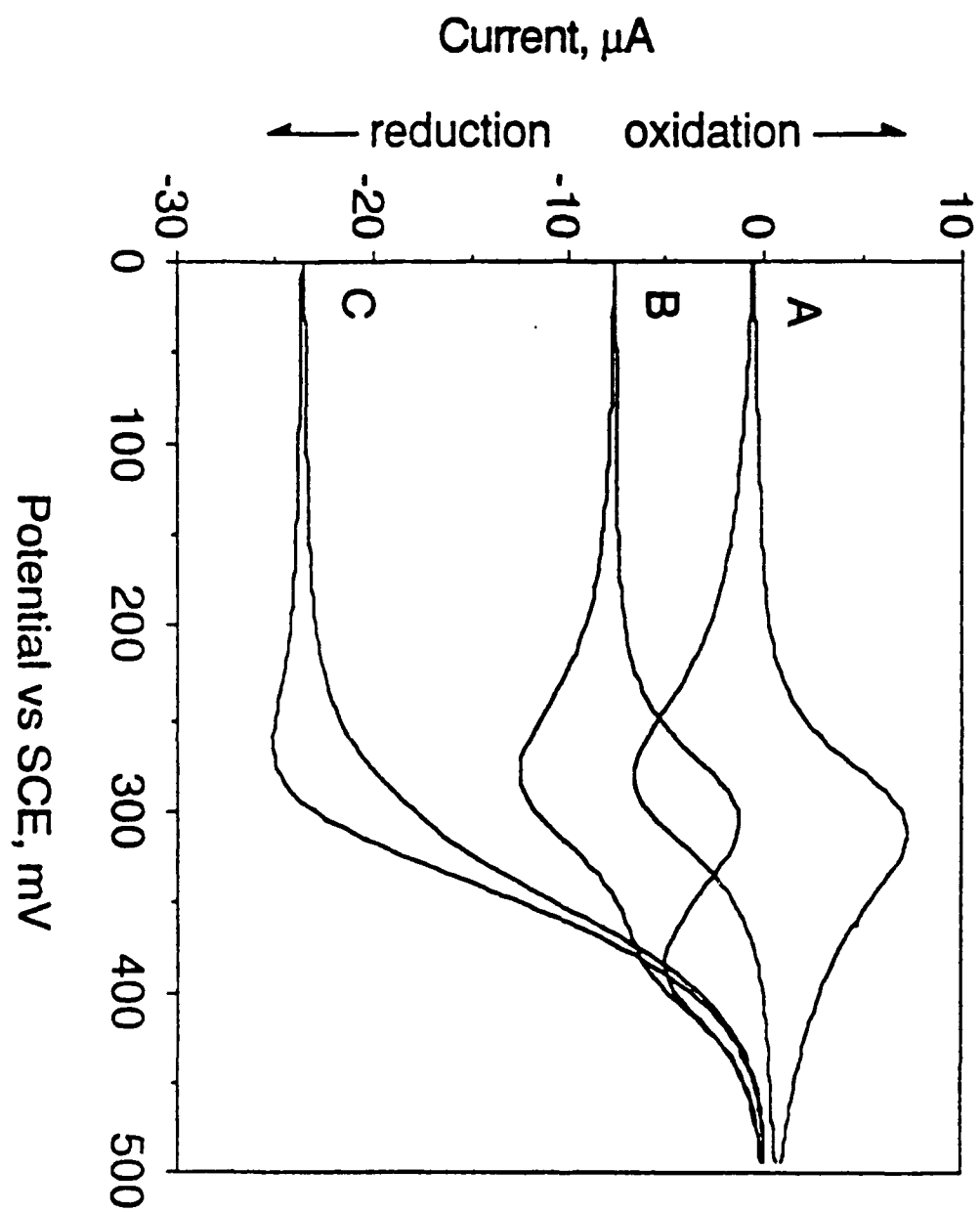


fig. 2





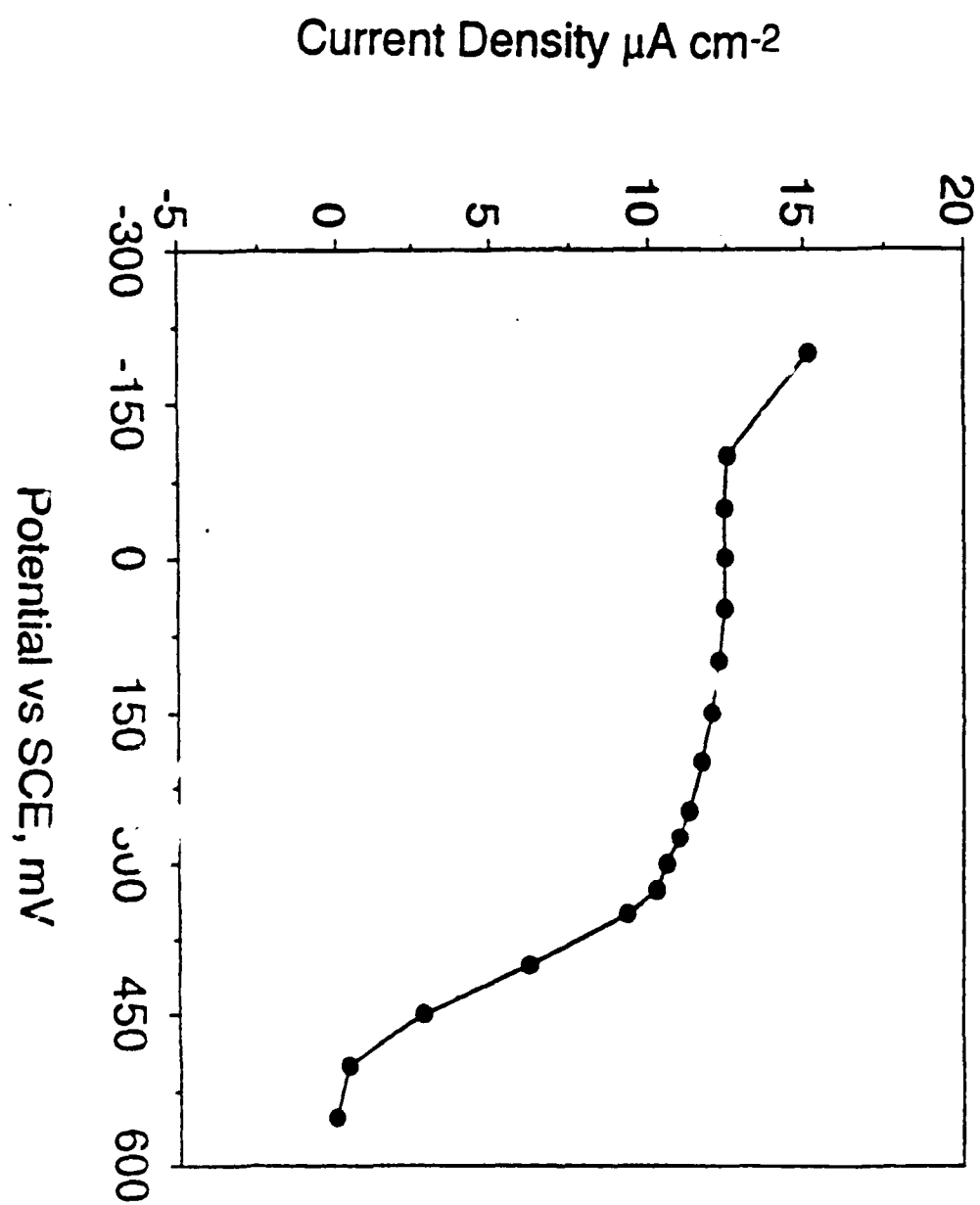
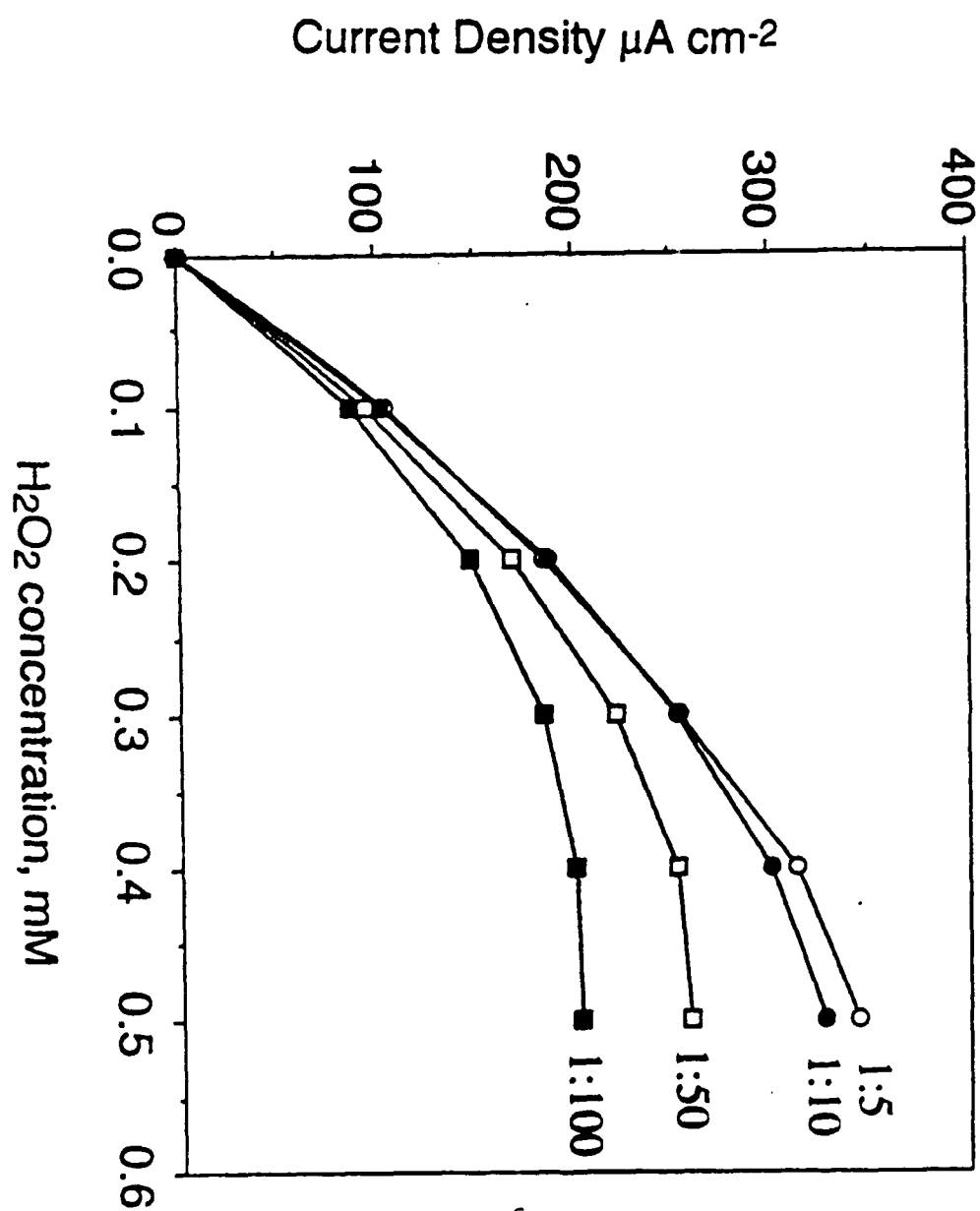
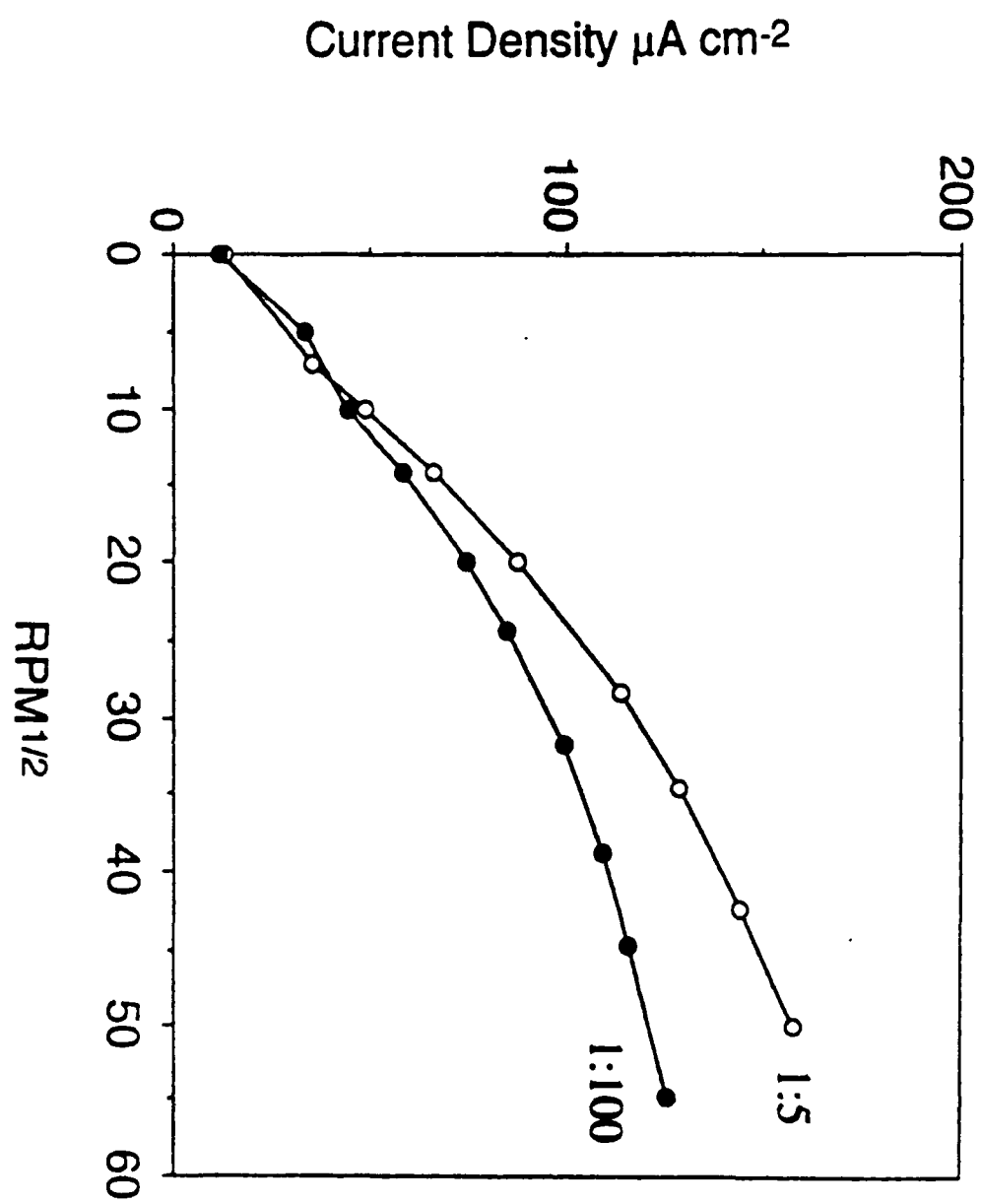
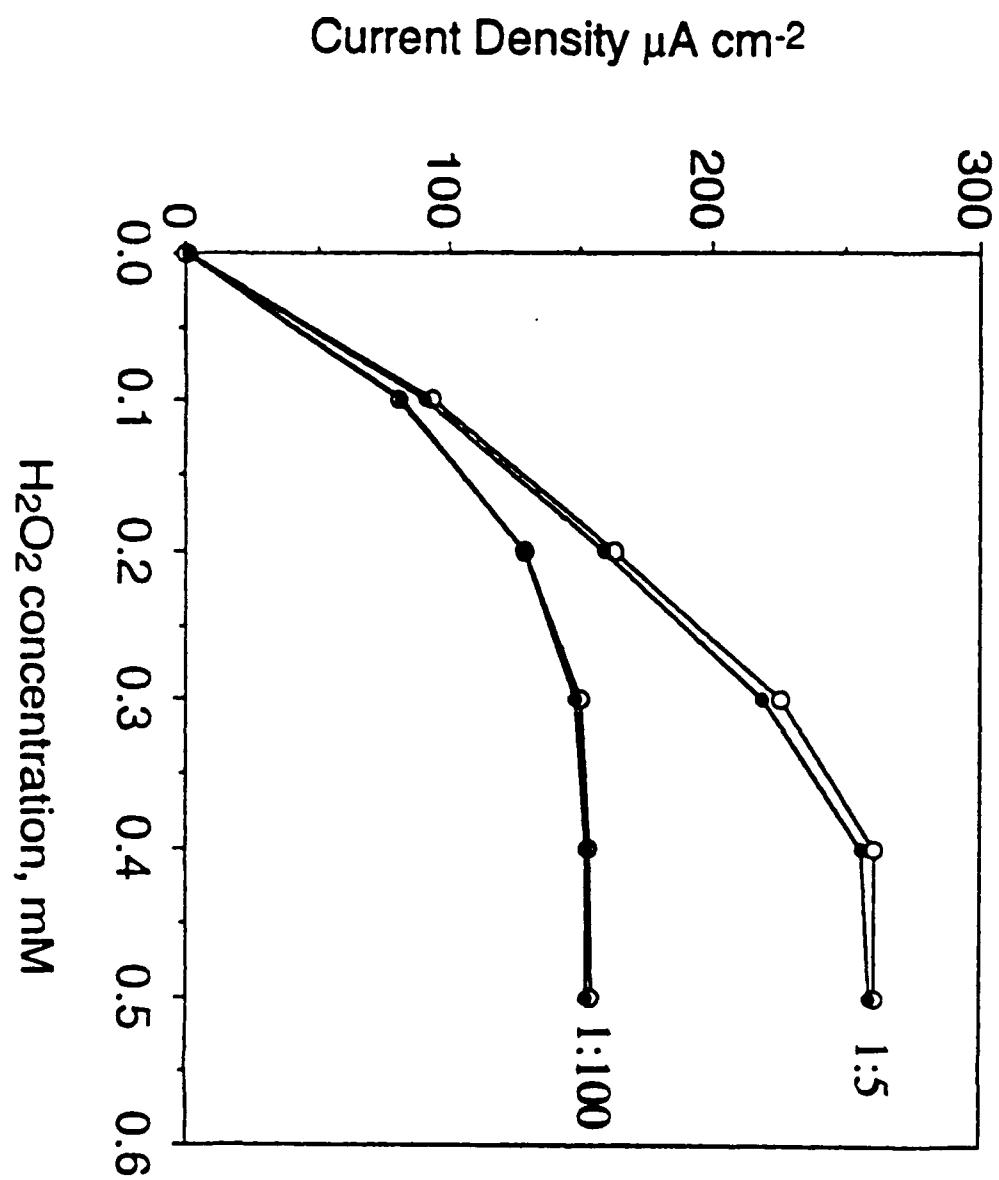
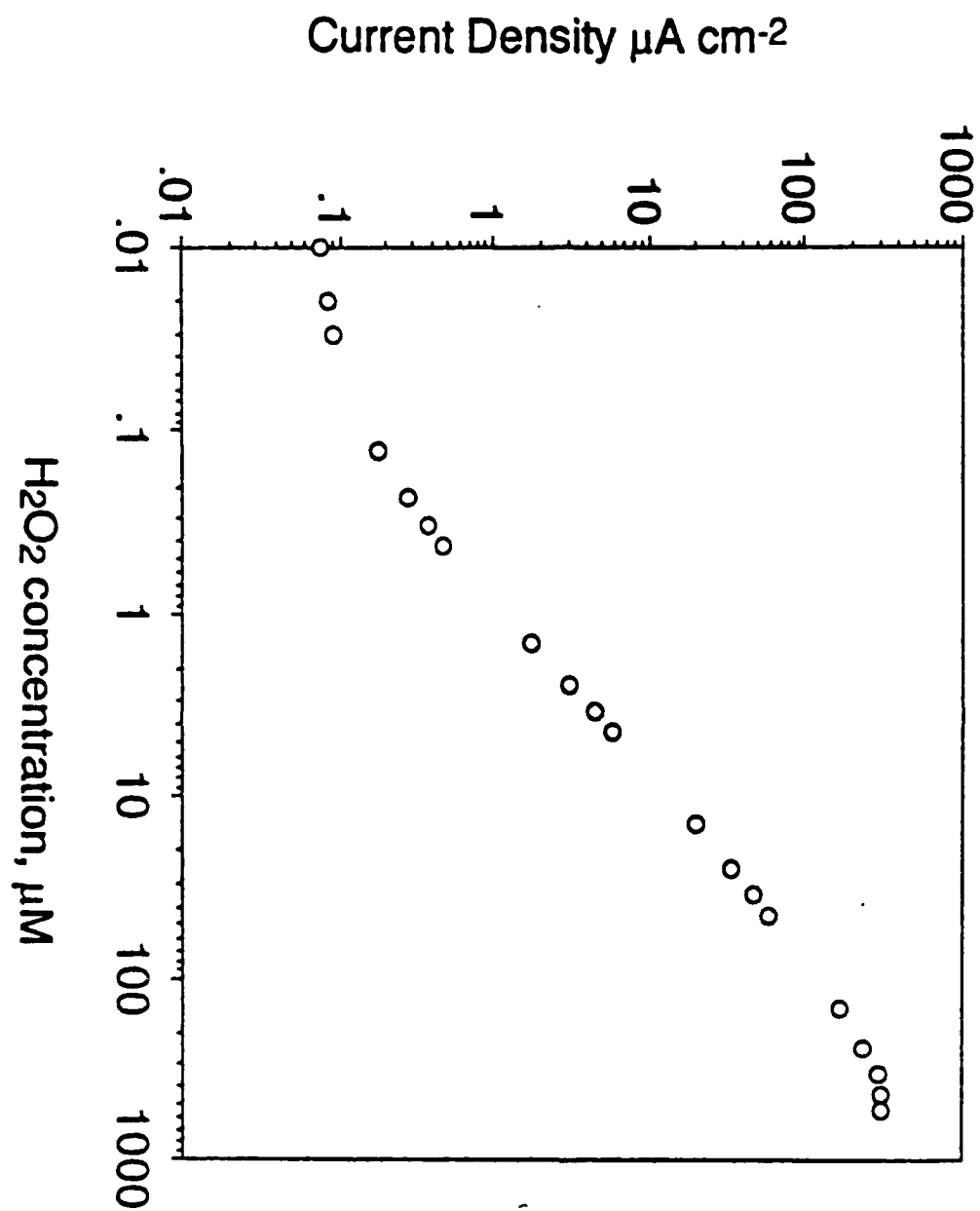


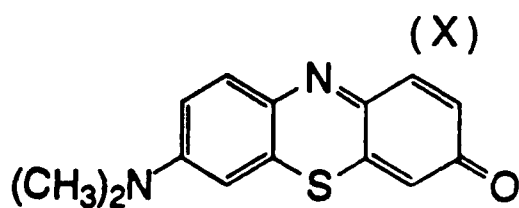
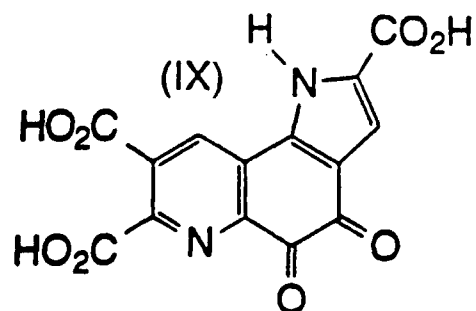
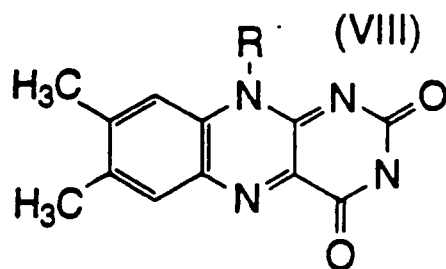
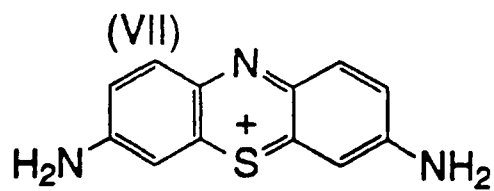
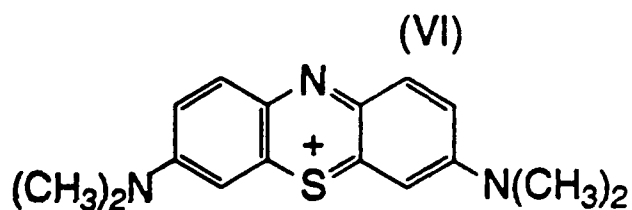
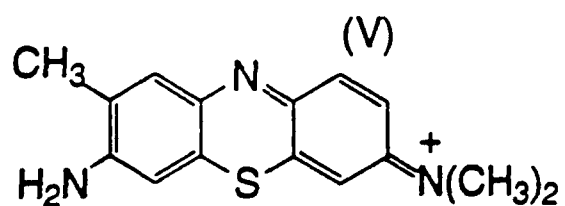
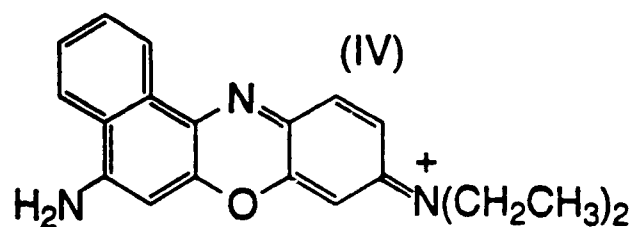
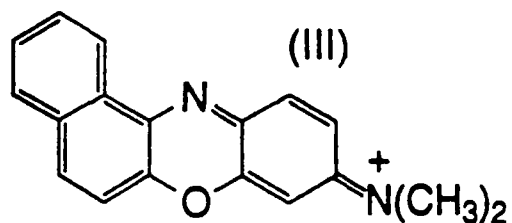
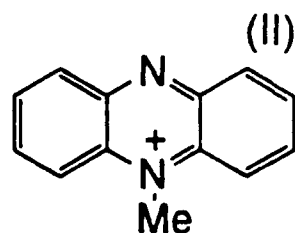
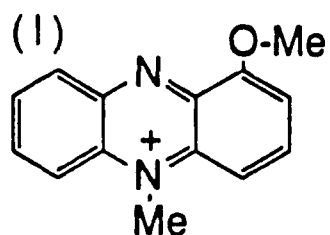
fig. 5

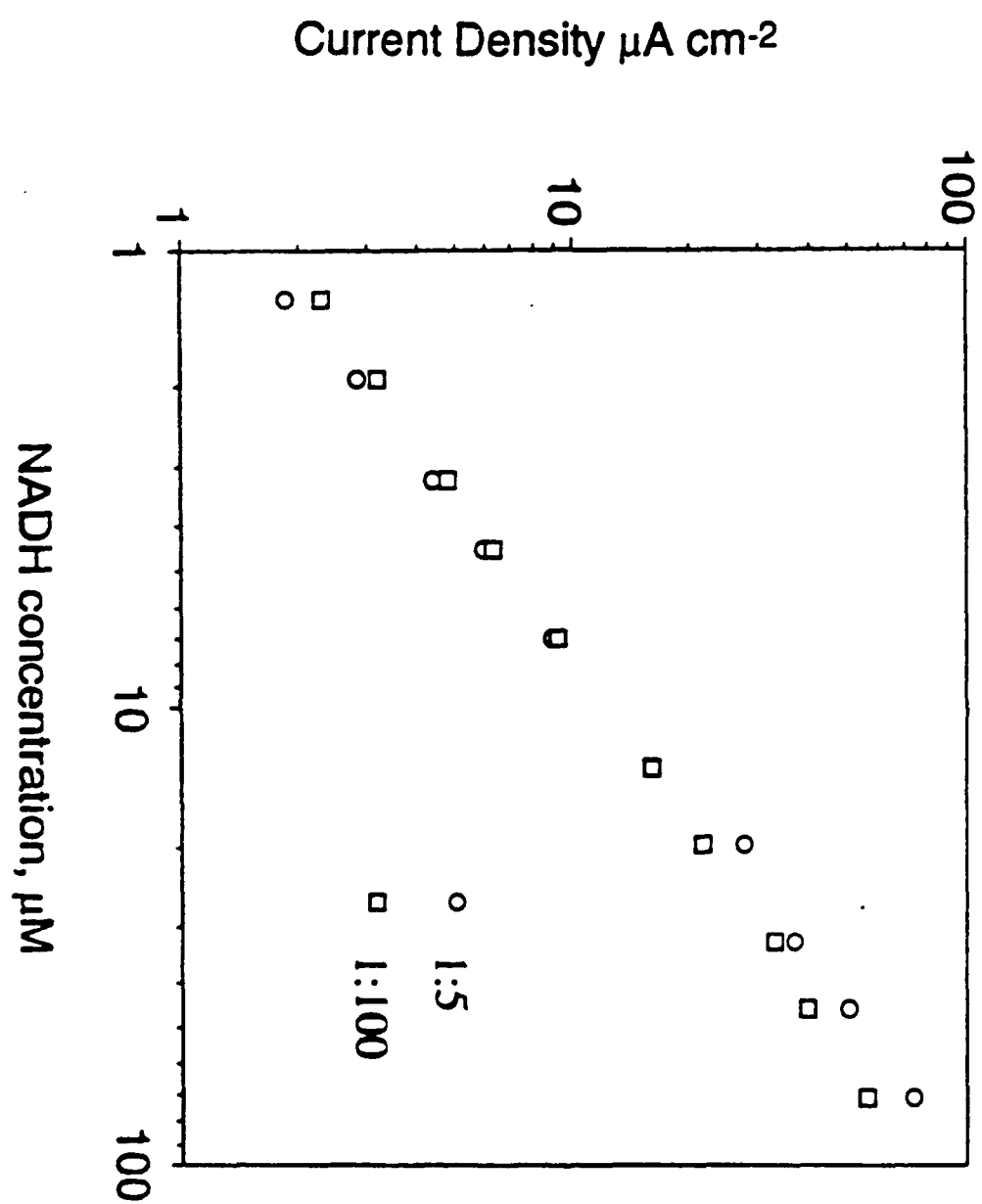


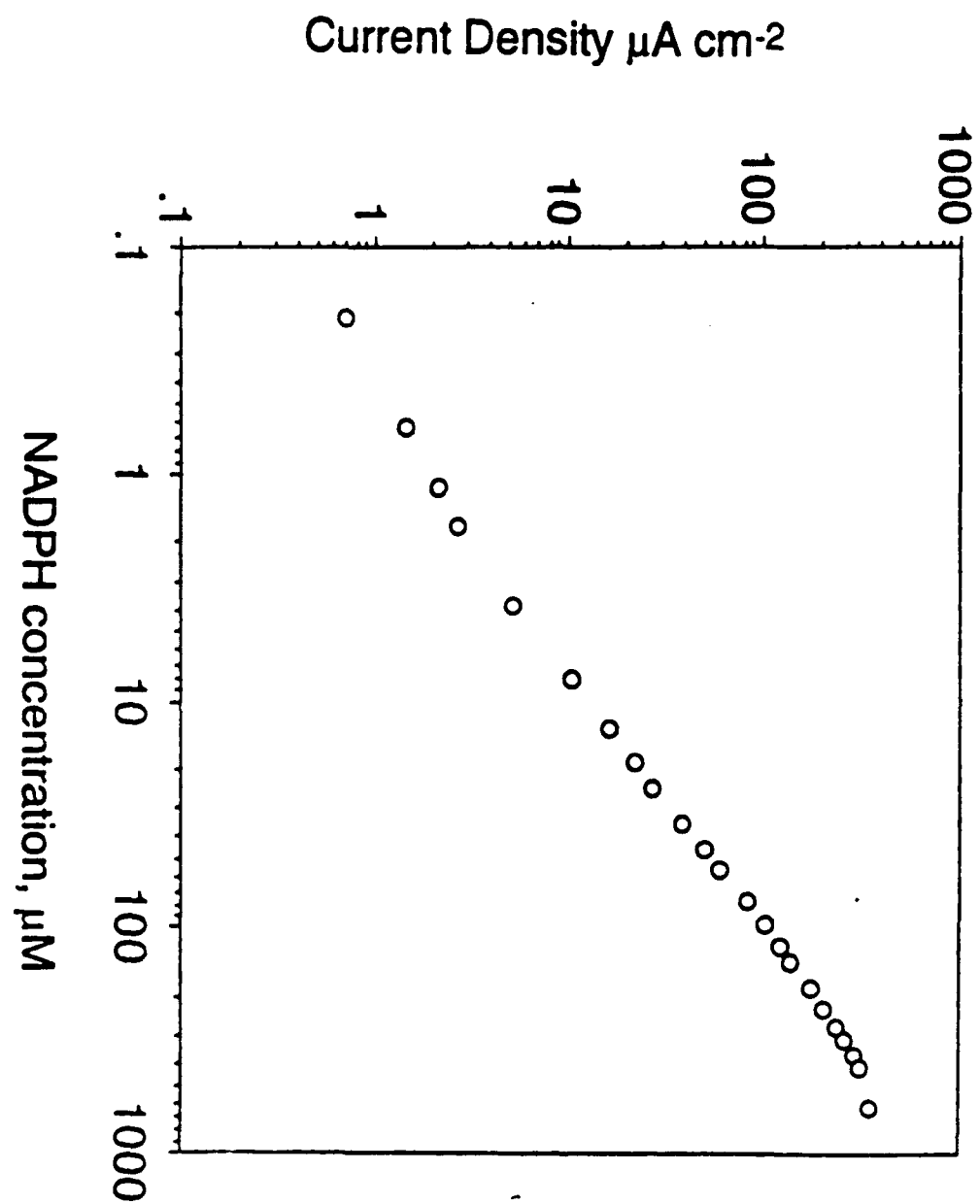












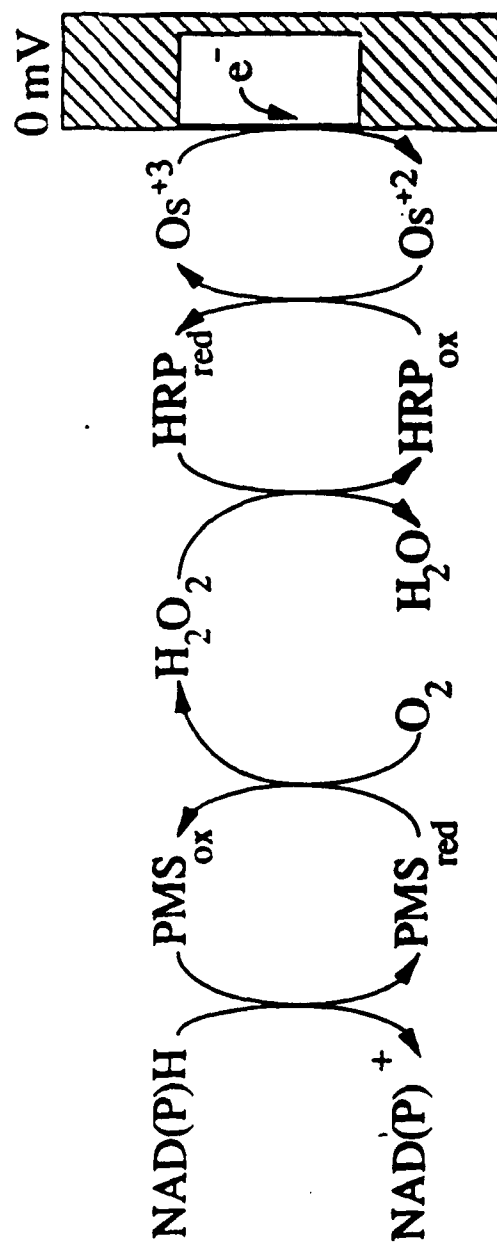


Table I: Amperometric H<sub>2</sub>O<sub>2</sub> Sensors Based on HRP Modified Electrodes

Electrode Surface	Mediator or Redox Matrix	Electrode Potential <sup>a</sup>	Sensitivity Ac <sup>-2</sup> M <sup>-1</sup>	Linear range $\mu$ M	Comments	Reference
Glassy carbon	None	0.0	10 <sup>-2</sup>	-----	HRP covalently bound to a hydrophilic epoxy network. Polyvinyl pyridine-derived polyamine crosslinked with PEGDE.	This work
Glassy carbon	Polymer I	0.0	1	0.1-100	HRP covalently bound to hydrophilic epoxy network. Polymer I crosslinked with PEGDE.	This work
Spectrographic graphite	None <sup>c</sup>	0.05	0.175	0.1-500	BSA with glutaraldehyde cross-linking	36
Carbon paste	O-phenylene-diamine <sup>d</sup>	-0.15	N.A. <sup>b</sup>	3.1-200	butanone peroxide was used as the substrate	37

Pt	Hexacyanoferrate (.01M) <sup>d</sup>	-0.05	Note e	5-1700	HRP was immobilized onto a nylon net	38
NMP+TCNQ <sup>-</sup>	None <sup>f</sup>	0.05	.168	----	HRP entrapped with dialysis membrane	39
SnO <sub>2</sub>	ferrocene carboxylic acid <sup>d</sup>	0.2	.04	0.01-1	HRP immobilized with glu- taraldehyde	40
Carbon paste	ferrocene <sup>d</sup>	0.05	N.A. <sup>b</sup>	0.1-10	Nafion coating was applied to the electrode to prevent loss of mediator	41
Graphite foil	potassium hexa- cyanoferrate(II) <sup>d</sup>	-0.02 <sup>a</sup>	0.03	<600	electrolyte was dioxane with 15% aqueous buffer	42
Carbon fiber <sup>g</sup>	None	Note h	-----	40-5000	The biotin/avidin complex was used to obtain a surface layer of HRP	43

Pt, organic metal, or glassy carbon	potassium ferrocyanide	Note i	-----	membrane with albumin and glutaraldehyde	44
Spectrographic graphite or Carbon film	hexacyano- ferrate (II) <sup>d</sup>	0.0	Note e	.1-1000 HRP immobilized on arylamino- derivatized controlled-pore glass, packed into a flow through reactor	45
Amino silylated glassy carbon	hexacyano- ferrate (II) <sup>d</sup>	0.0	Note i	----- Glycerophosphate oxidase, HRP and BSA were covalently cross- linked on the glassy carbon surface.	46
Glassy carbon	hexacyano- ferrate (II) <sup>d</sup>	0.0	Note i	----- Albumin, glutaraldehyde, HRP and oxidase (xanthine, uricase, glucose) matrix held close to the electrode with a dialysis membrane.	47
Gold or graphite	Several <sup>k</sup>	Note k	2.0 <sup>j</sup>	0.05-6 <sup>j</sup> HRP was free in solution	48

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a) potential vs SCE

b) macroporous electrode, the true surface area is unknown

- c) uncertainty as to whether surface species created during electrode pretreatment are mediating
- d) freely diffusing mediator
- e) flow system
- f) probably mediated by soluble component of organic metal or reaction product of organic metal
- g) microelectrode
- h) cyclic voltammetry used to provide selective detection of oxygen generated by autocatalytic decomposition of hydrogen peroxide
- i) HRP incorporated into a bienzyme system
- j) best reported result for ferrocene monocarboxylic acid
- k) mediators used and redox potential are:  $[\text{Ru}(\text{NH}_3)_5\text{py}](\text{ClO}_4)_3$  - +28 CpFeC<sub>2</sub>B<sub>9</sub>H<sub>11</sub> - -80 1,1'-dimethyl-3-(2-aminoethyl)ferrocene - +75 (2-aminoethyl)ferrocene - +185 Ferrocenemonocarboxylic acid - +275 aminomethylferrocene - +309mV

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